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Biodiversity and Classification of Lactococcal Phages

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For this study, an in-depth review of the classification of *Lactococcus lactis* phages was performed. Reference phages as well as unclassified phages from international collections were analyzed by stringent DNA-DNA hybridization studies, electron microscopy observations, and sequence analyses. A new classification scheme for lactococcal phages is proposed that reduces the current 12 groups to 8. However, two new phages (Q54 and 1706), which are unrelated to known lactococcal phages, may belong to new emerging groups. The multiplex PCR method currently used for the rapid identification of phages from the three main lactococcal groups (936, c2, and P335) was improved and tested against the other groups, none of which gave a PCR product, confirming the specificity of this detection tool. However, this method does not detect all members of the highly diverse P335 group. The lactococcal phages characterized here were deposited in the Félix d'Hérelle Reference Center for Bacterial Viruses and represent a highly diverse viral community from the dairy environment.

Strains of the gram-positive bacterium *Lactococcus lactis* are used by the dairy industry to acidify milk during the manufacture of fermented products, such as cheese, buttermilk, and sour cream. The use of various *L. lactis* strains is essential for controlling virulent phages that are responsible for most milk fermentation collapses (40). Lactococcal phages are ubiquitous in the dairy environment, as they are found in raw milk and survive pasteurization (14, 36). Due to their negative effects on fermentation as well as their biodiversity within this ecological niche, numerous lactococcal phages have been isolated and characterized, with the overall aim of improving phage control strategies. Currently, only coliphages have received more attention than lactococcal phages (1, 2). All known *L. lactis* phages have a double-stranded genome and a noncontractile tail. According to the International Committee on Taxonomy of Viruses, *L. lactis* phages are members of the *Caudovirales* order, an extremely large, morphologically and genetically diverse group that encompasses over 95% of all known phages (37). This order contains three families, namely, the *Myoviridae* (with long, contractile tails), the *Siphoviridae* (with long, noncontractile tails), and the *Podoviridae* (with short tails). Lactococcal phages are mainly members of the *Siphoviridae* family, with a few members from the *Podoviridae* family.

Over a decade ago, a classification scheme which was mainly based on phage morphology and DNA homology criteria was developed for lactococcal phages (3, 4, 7, 25, 32). It is made up of 12 lactococcal phage groups and has been used successfully for comparing lactococcal phages isolated from around the world. It rapidly became obvious that the vast majority of lactococcal phages belong to one of three main groups, the 936, c2, and P335 groups. Consequently, most studies of lactococcal phages have dealt with these groups. For example, a multiplex PCR method is now available to rapidly assign newly isolated phages to one of these three main groups (29). Moreover, 14 complete lactococcal phage genomic sequences are now available in a public database (www.ncbi.nlm.nih.gov), including 10 from the P335 group, two from the c2 group, and two from the 936 group. The genome of phage asccϕ28, from a fourth species (P034), was also reported a few years ago (27, 28), but the sequence is not yet available. Many other lactococcal phage genome projects are also under way.

The cataloging scheme for lactococcal phages has been under scrutiny lately due to the comparative analysis of an increasing number of genomic sequences and the recurring emergence of new virulent phages (13, 42). For instance, comparative genome analysis has led to a proposal to merge the BK5-T species with the P335 species, reducing the number of lactococcal phage species to 11 (30). Based on DNA-DNA hybridization studies, others have also suggested including the 1483 and T187 species in the P335 group (24, 30, 43, 47).

Despite the strategies developed to control phages, this biotechnological problem still remains the most common cause of slow or incomplete milk fermentation. The biodiversity and evolution of phage populations are partly responsible for the difficulty in controlling them. Phage populations in dairy factories are also in a dynamic state, and their composition must be monitored closely to ensure the efficacy of the current control strategies that are based, in part, on the phage species sensitivity of *L. lactis* strains (40, 42). Consequently, the aim of this study was to reassess the classification system for *L. lactis* phages.

MATERIALS AND METHODS

Bacterial strains, phages, and media. The *Lactococcus lactis* strains and bacteriophages used in this study are listed in Table 1. Strains were grown at 30°C in M17 broth (51) supplemented with 0.5% glucose (GM17) (Quélab) unless otherwise specified. All phages were propagated from a single plaque as de-

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TABLE 1. Bacteriophages and host strains used in this study

Family	Phage	Species		*L. lactis* host strain	Reference	Source
		New	Old[a]			
Siphoviridae	bIL170	936	936	IL1403	15	U. Laval[c]
	c2	c2	c2	LM0231	35	U. Laval[c]
	CB17	c2	Unclassified[b]	SMQ-436	This study	This study
	GR6	c2	Unclassified[b]	SMQ-361	This study	This study
	1483	P335	1483	111	23, 24	U. Laval[c]
	r1t	P335	P335	R1K10	34, 55	U. Laval[c]
	T189	P335	T187	205.RV	47	B. Geller[d]
	ul36	P335	P335	SMQ-86	30	U. Laval[c]
	BK5-T	P335	BK5-T	H2	17	F. K. Vogensen[e]
	949	949	949	ML8	23	U. Laval[c]
	bIL168	949	Unclassified[b]	IL-16		This study
	P087	P087	P087	C10	7, 32	H. Neve[f]
	1358	1358	1358	582	23	U. Laval[c]
	1706	1706	Unclassified[b]	SMQ-450	This study	C. Fremaux[g]
	Q54	Q54	Unclassified[b]	SMQ-562	This study	This study
Podoviridae	1138	P034	Unclassified[b]	SMQ-450	This study	C. Fremaux[g]
	P369	P034	P034	F7/2	7, 32	H. Neve[f]
	KSY1	KSY1	KSY1	IE-16	50	U. Laval[c]

[a] Species from previous classification (25).
[b] Unclassified by multiplex PCR (29).
[c] Félix d'Hérelle Reference Center for Bacterial Viruses, Université Laval, Québec, Canada (www.phage.ulaval.ca).
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[f] Institute of Microbiology, Federal Dairy Research Center, Kiel, Germany.
[g] Danisco, Dangé Saint-Romain, France.

scribed previously (19). High phage titers were obtained by using the method of Jarvis (22). To obtain maximal titers and/or visible plaques, some phages (1483, 949, P087, P369, KSY1, and 1706) and their respective hosts were incubated at 22°C for 24 h. Glycine (0.5%) was also added to the top agar to increase plaque size and facilitate phage enumeration (33). The induction of lactococcal prophages was performed as described previously (41). When needed, phage lysates were concentrated with polyethylene glycol (12, 22, 23) and purified on a discontinuous-step CsCl gradient (49). Ultracentrifugation was performed using a Beckman SW41 Ti rotor at 35,000 rpm for 3 h.

Phage DNA analysis. The genomic DNAs of phages ul36, r1t, 1706, Q54, c2, and bIL170 were isolated by using Lambda Maxi DNA purification kits (QIAGEN), with previously described modifications (18). The DNAs of phages P369, 1483, 1358, 949, 1138, P087, and KSY1 were isolated from CsCl-purified phages as reported elsewhere (12). Lastly, the DNAs of phages T189, bIL168, BK5-T, GR6, and CB17 were isolated by using a previously described protocol (41). Restriction endonucleases (Roche Diagnostics) were used as recommended by the manufacturer. After restriction, phage DNA samples were heated for 10 min at 70°C to avoid possible cohesive end ligation. The DNA fragments were separated in 0.8% agarose gels in 1× Tris-acetate-EDTA buffer and visualized by UV photography after being stained with ethidium bromide. The phage DNAs were transferred to positively charged nylon membranes (Roche Diagnostics) by capillary blotting as described by Sambrook and Russell (49). Phage genomic DNAs used as probes were randomly labeled with Dig High-Prime labeling kits (Roche Diagnostics). Prehybridization, hybridization, washes, and detection by chemiluminescence (CDP-star) were performed as suggested by the manufacturer (Roche Diagnostics).

Electron microscopy. For electron microscopy, 1.5 ml of phage lysate (10^8 to 10^9 PFU/ml) was centrifuged for 1 h at 4°C (24,000 × *g*). The supernatant (approximately 1.4 ml) was gently discarded. The remaining lysate was diluted twice by the addition of 1 ml of ammonium acetate (0.1 M, pH 7.5) and then centrifuged (1 h at 24,000 × *g* and 4°C). The phage solution (15 μl) was mixed with the stain (15 μl of 2% phosphotungstic acid, pH 7.5) on a nickel Formvar-carbon-coated grid (Pelco International). The liquid was removed after 1 min by touching the edge of the grid with blotting paper. Phage morphology was observed by using a JEOL 1230 transmission electron microscope at 80 kV. Dimensions of the phages are the means of at least 10 specimens.

PCR and DNA sequencing. For multiplex PCR, the DNA template was made up of a phage lysate treated with DNase (final concentration, 1 μg/ml) for 30 min at 37°C. The conditions and primers used were previously described (29). PCR products corresponding to the genes coding for the major capsid proteins (*mcp*) of the c2-like phages GR6 and CB17 were sequenced on both strands by using specific oligonucleotides (29) and an ABI Prism 3700 apparatus from the genomic platform at the Centre Hospitalier de l'Université Laval. The 16S rRNA genes were amplified from *L. lactis* strains SMQ-450 and SMQ-562, using the *Bacteria*-specific primers SSU27F (5′ AGAGTTTGATCMTGGCTCAG 3′) and SSU1492R (5′ TACGGYTACCTTGTTACGACTT 3′).

Bioinformatic analysis. Computer-assisted DNA analyses were performed by using version 10.3 of the Genetics Computer Group sequence analysis software package and the ClustalW website (http://www.ebi.ac.uk/clustalw/). PSI-BLAST and Advanced BLAST Search 2.1 were also used for sequence comparisons with databases (5).

Nucleotide sequence accession numbers. The complete genomic sequences of the P335-like phages analyzed in this study are available under the following GenBank accession numbers (phage name [accession number]): 4268 (AF489524), bIL285 (AF323668), bIL286 (AF323669), bIL309 (AF323670), BK5-T (AF176025), phiLC3 (AF242738), r1t (U38906), TP901-1 (AF304433), Tuc2009 (AF109874), and ul36 (AF349457). For the c2-like phages, in addition to the complete genomic sequences of phages c2 (L48605) and bIL67 (L33769), the nucleotide sequences of the genes coding for the major capsid protein are available for phages eb1 (AF152410), Q44 (AF152412), Q38 (AF152411), CB17 (DQ110947), and GR6 (DQ110948). Complete genomic sequences are available for the 936-like phages sk1 (NC_001835) and bIL170 (NC_001909).

RESULTS

Selection of lactococcal phages. As a first step, a representative of each of the 12 previously recognized lactococcal phage species was obtained (Table 1). Whenever possible, we selected phages for which the complete genomic sequence was already available. Seven reference phages (949, 1358, 1483, c2, KSY1, bIL170 [936 species], and ul36 [P335 species]) were obtained from the Félix d'Hérelle Reference Center for Bacterial Viruses (www.phage.ulaval.ca) at Université Laval (Quebec City, Canada). Phage T189 was provided by B. Geller (Oregon State University) and was used as a representative of

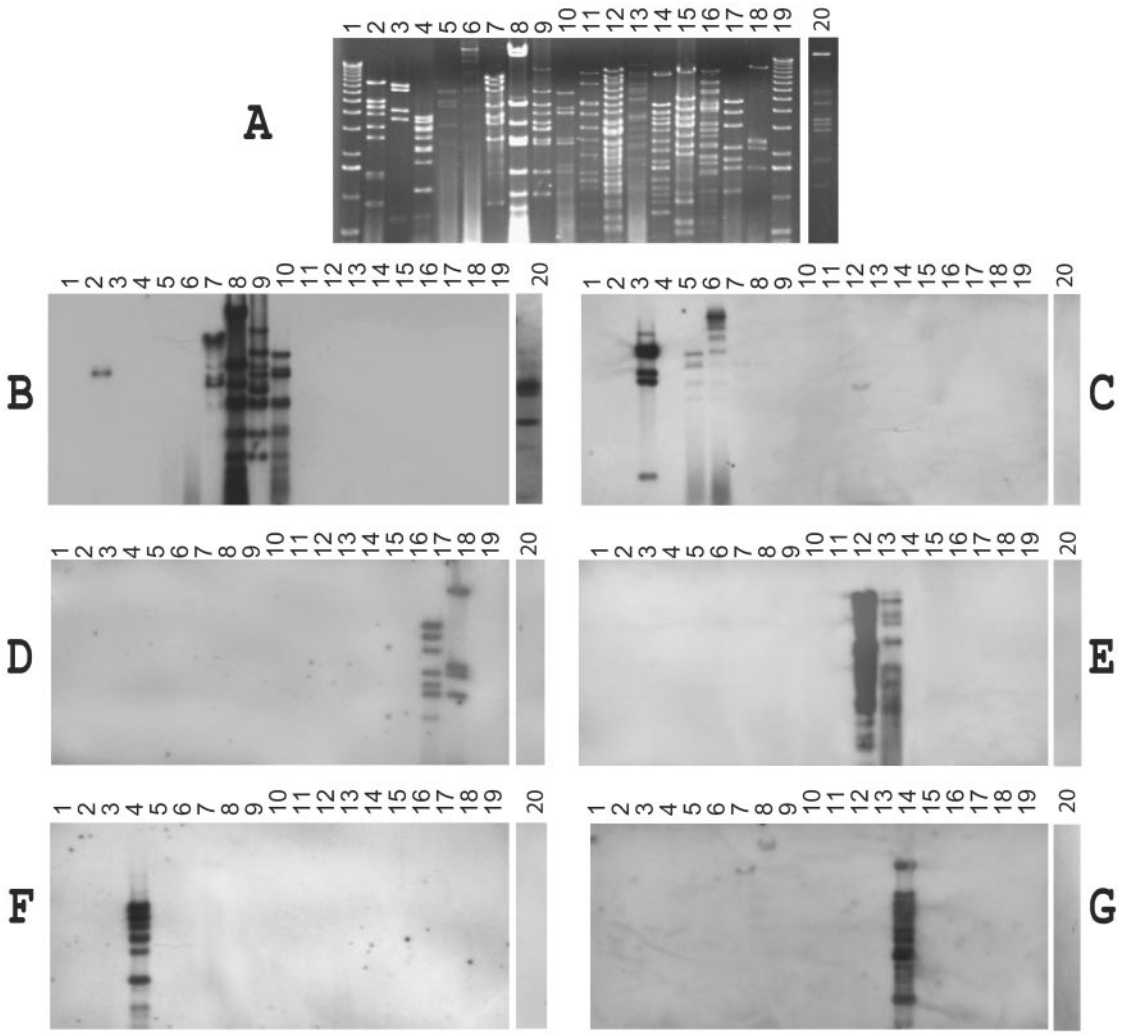


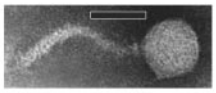
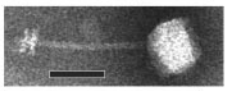
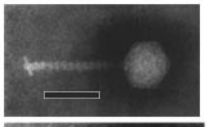
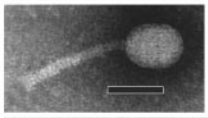
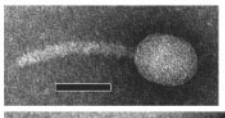
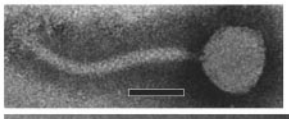
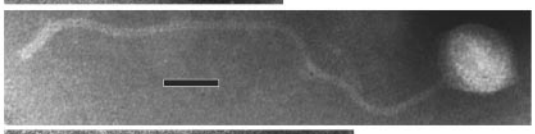
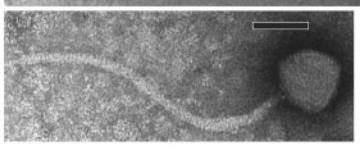
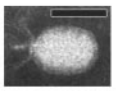
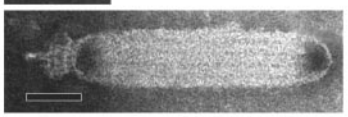
FIG. 1. (A) Restriction profiles of lactococcal phages analyzed in this study. (B to G) Analysis of phage genomic DNAs by Southern hybridization, using the complete genomes of the following phages as probes: panel B, r1t; panel C, c2; panel D, P369; panel E, 949; panel F, Q54; panel G, 1706. Lanes 1 and 19, 1-kb DNA ladder (Invitrogen); lanes 2, phage bIL170 (genome digested with EcoRV); lanes 3, c2 (EcoRI); lanes 4, Q54 (EcoRI); lanes 5, GR6 (EcoRI); lanes 6, CB17 (EcoRI); lanes 7, ul36 (EcoRV); lanes 8, r1t (EcoRV); lanes 9, 1483 (EcoRV); lanes 10, T189 (EcoRV); lanes 11, P087 (EcoRV); lanes 12, 949 (EcoRV); lanes 13, bIL168 (EcoRV); lanes 14, 1706 (EcoRV); lanes 15, 1358 (EcoRI); lanes 16, KSY1 (AsnI); lanes 17, P369 (EcoRV); lanes 18, 1138 (EcoRV); lanes 20, BK5-T (EcoRV).

the T187 species since phage T187 is no longer available. Phages P087 and P369 (P034 species) were provided by H. Neve (Institute of Microbiology, Federal Dairy Research Center, Germany), and phage BK5-T was provided by F. K. Vogensen (KVL University, Denmark). Unfortunately, we could not obtain a viable representative of the P107 species, not even from the laboratory that originally isolated this phage (32). All of the samples provided failed to generate the restriction profile for P107 (7), and all of the P107 lysates contained a phage that belonged to the 936 species (data not shown). This lactococcal reference phage thus appears to be extinct. We also included prophage r1t as another representative of the P335 species because it shares only 11% nucleotide sequence identity with the virulent P335-like phage ul36 (30). Lastly, six unclassified lactococcal phages (bIL168, CB17, GR6, Q54, 1138, and 1706) obtained from various collaborators were also included in this study. Whenever possible, the DNA restriction profiles of these phages were compared to published profiles to confirm their identities.

Analysis of genomic DNA by Southern hybridization. Digoxigenin-labeled genomic probes were produced from each of the previously described lactococcal species and were hybridized with the restricted genomes of the 18 phages studied. To take into account the genetic diversity previously observed within the P335 species (30), a genomic probe was made from each of the two representatives (r1t and ul36) of this group.

Phages bIL170, KSY1, P087, and 1358 only hybridized with themselves (data not shown), which is in agreement with previous reports (7, 23, 25). Phages 1483, T189, and BK5-T (Fig. 1B) shared homology with the P335 phages r1t and ul36. A weak hybridization signal of the r1t probe with the restricted genome of bIL170 (936 species) was also noted (Fig. 1B). Based on the complete genomes of bIL170 (15) and r1t (55), this area was identified as a 950-bp region that shares 91% nucleotide se-

TABLE 2. Biodiversity of bacteriophages infecting *Lactococcus lactis*

Family Species	Phage	Capsid diameter (nm)	Tail width (nm)	Tail length (nm)	Electron micrograph[a]
Siphoviridae					
936	bIL170	50	11	126	
P335	ul36	49	7	104	
1358	1358	45	10	93	
c2	c2	54 X 41	10	95	
Q54	Q54	56 X 43	11	109	
P087	P087	59	14	163	
949	949	70	12	490	
1706	1706	58	11	276	
Podoviridae					
P034	P369	57 x 40	5	19	
KSY1	KSY1	223 X 45	6	32	

[a] Bars, 50 nm.

quence identity and codes for the neck-passage structure protein (9). The significance of this protein remains to be investigated, as not all members of these two phage groups possess this morphological feature. The c2 genomic probe strongly hybridized with the genomes of the unclassified phages CB17 and GR6 (Fig. 1C). The P369 probe strongly hybridized with the genome of phage 1138 (Fig. 1D). Likewise, the genome of phage bIL168 clearly hybridized with the 949 probe (Fig. 1E).

None of the probes from the known lactococcal phage species hybridized with the genome of phage Q54 or 1706. A Q54 probe and a 1706 probe also failed to hybridize with the other lactococcal phage genomes (Fig. 1F and G, respectively).

Phage morphology. The 18 lactococcal phages were observed by transmission electron microscopy to determine their morphology (Table 2). Some of these phages have already been characterized, and our observations were in agreement with published data (3, 7, 23, 25). Fifteen phages had long, noncontractile tails and belonged to the *Siphoviridae* family, while three phages (P369, KSY1, and 1138) had short tails and belonged to the *Podoviridae* family. Phages sharing DNA-DNA

homology had the same morphotype (1), but some morphological features (tail length and the presence of a collar structure or baseplate) varied slightly. For example, phage bIL170 has a collar structure that is absent from phage sk1 (data not shown). Only one additional gene is necessary for the presence of this structure (11, 15). Moreover, it is known that the tape measure protein determines the tail length and that a mutation in its gene can lead to tail size variations (45, 56).

Phage 1706, which shares no homology with the other phages analyzed in this study, has a morphology not previously reported for lactococcal phages (Table 2). The capsid size of phage 1706 is similar to that of P087, but its noncontractile tail is longer. On the other hand, the morphology of phage Q54 resembles that of phage c2. The host strains for these phages, *L. lactis* SMQ-450 (host for 1706) and *L. lactis* SMQ-562 (host for Q54), are used commercially in mesophilic starter cultures to make fermented milk products. The identities of the bacterial species were confirmed by 16S rRNA gene sequencing (data not shown). Moreover, host range analyses revealed that *L. lactis* SMQ-450 was sensitive to the lactococcal phages 1706 and 1138, while *L. lactis* SMQ-562 was sensitive to lactococcal phages Q54 and 949.

Based on DNA-DNA hybridization and morphology results, 16 of the phages studied here could be classified into eight groups (Table 1). The two remaining phages (1706 and Q54), which were unrelated to the others, might be members of new emerging groups.

Multiplex PCR test for the identification of phages. As indicated previously, a multiplex PCR method is available to identify the three predominant lactococcal phage groups for which the sequences of several members are known, namely, 936, c2, and P335-like phages (29). The accuracy of this method was checked with the 18 selected phages (Fig. 2). As expected, PCR amplicons of the predicted sizes were obtained for the reference phages c2, bIL170 (936 species), and r1t (P335 species). Interestingly, phage 1483 gave a PCR product with a size expected for a P335-like phage. This observation, together with DNA-DNA hybridization results (Fig. 1B), suggested that it may belong to the P335 group (Fig. 2). We failed to obtain a PCR product with the other 14 phages. The negative PCR results with phages ul36 and BK5-T (P335 species) have been discussed in the past and are due to the absence of the target region in their genome (29, 30).

Surprisingly, the multiplex PCR method (29) failed to identify phages CB17 and GR6 as members of the c2 species. The two primers (c2A and c2B) used to amplify a 474-bp product specific to c2-like phages are based on two conserved regions (20 nucleotides) in the gene coding for the major capsid protein. The regions covering both primers were sequenced in phages CB17 and GR6 and compared with similar sequences in public databases (Fig. 2C). The c2A primer regions in phages CB17 and GR6 were identical to the same regions for other c2-like phages. However, the c2B primer contained three nucleotide mismatches (Fig. 2C). Of particular interest was a mismatch at the 3′ end that corresponds to the wobble base of the serine codon. A new oligonucleotide (c2C) was designed in which the 20-mer was shifted one nucleotide to avoid the 3′ codon base. The multiplex PCR method was retested using the c2A/c2C primer pair. The three c2-like phages (c2, CB17, and GR6) generated 475-bp PCR products, while the 14 lactococcal phages of the other species did not generate PCR products (Fig. 2B). To confirm the efficacy of this new set of c2-specific primers, 13 other distinct c2-like phages (bIL67, Q44, eb1, ml3, HD1, HD2, HD4, HD7, HD25, HD26, GR3, GR4, and CB27) were tested. PCR products of the expected size were obtained from all of them (data not shown).

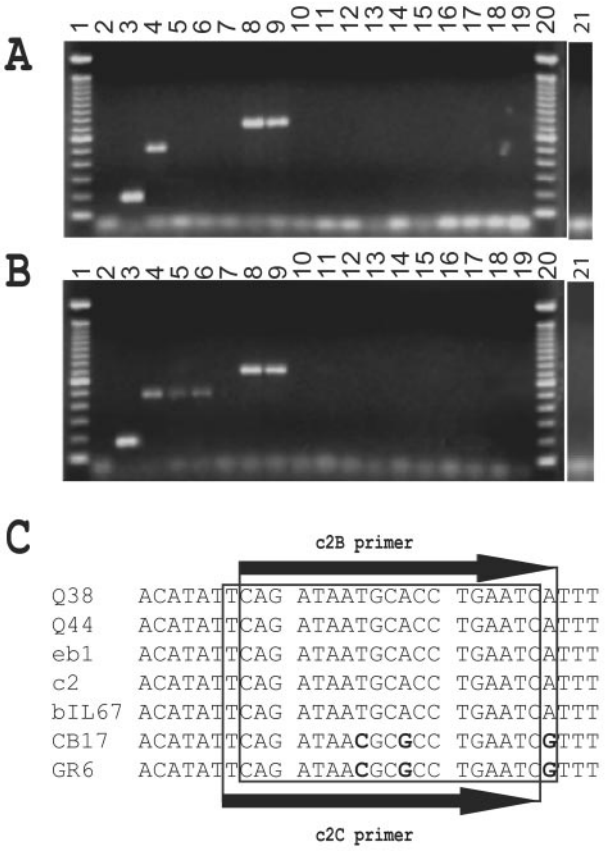


FIG. 2. Identification of lactococcal phage species by multiplex PCR. (A) Three pairs of previously described primers (29) were used for PCR. (B) The same three sets of primers were used, except that the c2B primer was replaced by the c2C primer. Lanes 1 and 20, 100-bp DNA ladder (Invitrogen); lanes 2, negative control; lanes 3, bIL170; lanes 4, c2; lanes 5, GR6; lanes 6, CB17; lanes 7, Q54; lanes 8, r1t; lanes 9, 1483; lanes 10, ul36; lanes 11, T189; lanes 12, P087; lanes 13, 949; lanes 14, bIL168; lanes 15, 1706; lanes 16, 1358; lanes 17, KSY1; lanes 18, P369; lanes 19, 1138; lanes 21, BK5-T. (C) Analysis of the region covered by primers c2B and c2C in c2-like phages for which the gene coding for the major capsid protein is available in GenBank.

DISCUSSION

The lactococcal phage classification system was first set up to characterize these phages and to provide better ways to control phage infections. This pragmatic classification (7, 25) has been applied universally and has led to comparisons of lactococcal phage collections worldwide, providing valuable results. However, more sequence data, together with the isolation of new phages and the absence of an international collection housing reference phages, led us to revise the previous classification and to deposit all the phages used in this study in the Félix d'Hérelle Reference Center for Bacterial Viruses

(www.phage.ulaval.ca). In our opinion, this phage set represents the best-characterized bank of viruses that share a well-defined ecological niche and that infect a single bacterial species.

Of the 12 phage groups previously described (7, 25), one appeared extinct (P107), while three others (1483, T187, and BK5-T) were merged with the P335 group, reducing the number of lactococcal phage groups from 12 to 8. Nonetheless, additional lactococcal phage groups may exist. For example, phages 1706 and Q54, which were unrelated to the other phages, could be members of two novel groups. Genomic analysis of these new phages is currently under way and will likely shed light on their origins and relationships with known lactococcal phages.

As indicated above, no member of the P107 species could be retrieved from any collection and thus could not be analyzed in this study. Unfortunately, this is not the first time that a lactococcal phage has been lost. Two previous studies reported the isolation of three phages of the *Myoviridae* family that infect *L. lactis*. Phage RZh was described prior to 1970 but has since been lost (53). The second lactococcal myophage was named c10III, but this classification remains uncertain because no contracted sheath was observed (26). In fact, the description and electron micrographs of phage c10III are very similar to those of phage P087 used in the study reported here, and both phages infect the same *L. lactis* strain (C10). However, since phage c10III is no longer available, the hypothetical relationship with phage P087 could not be investigated. A third putative lactococcal phage from the *Myoviridae* family was observed by transmission electron microscopy but could not be propagated (52). In the same study, a rare phage with a C1 morphotype (isometric capsids and a short tail) was also observed but was not amplified (52). The loss of these phages points to the need to deposit key phage representatives in public repositories and, ideally, in more than one collection around the world to ensure continued access to them and the reproducibility of future studies.

Three phage groups were merged with P335 due to the high degree of genome mosaicism in this species. Sequencing data revealed that P335 phages have variable genomes that share only 10 to 33% homology (13, 30). This gradient of genetic diversity is likely the consequence of recombination within an infected cell of the genomes of incoming lytic phage and prophage (6, 41). Some members (such as phage ul36) are particularly prone to such recombination (6, 41). This genome plasticity is likely a way to adapt to a new environment including a new host and new phage resistance mechanisms. From a classification point of view, DNA-DNA hybridization (Fig. 1) and a comparative analysis of the 10 P335-like genomes (Fig. 3) showed that the P335-like phages are a perfect example of a polythetic species. Indeed, the P335 species is composed of interconnected isolates with shared properties or modules. However, no single attribute is shared by all known members of this species. Such a mosaic structure makes it unfeasible to detect all the members of this species by using either a single phage genome as a probe for DNA-DNA hybridization or a single pair of primers for PCR assays (30). Previously, it was suggested that a dUTPase gene (Fig. 3) could be used as a target for the PCR detection of P335 phages because, at the time, it was found in all genomes of P335-like phages for which the complete sequence was available (30). However, no dUTPase gene was found in the recently analyzed genome of the virulent phage 4268 (P335 species).

The above results are indicative of the great biodiversity of lactococcal phages. However, phages from genetically different groups do not have the same commercial importance. Three predominant groups (936, c2, and P335) account for 98% of known lactococcal phages and are responsible for most dairy fermentation breakdowns (39, 40). One could speculate on why members of these three lactococcal phage groups predominate in this ecological niche. Clearly, the systematic use of selected *L. lactis* hosts with specific industrially relevant properties has led to the amplification of these phages in dairy factories. Some of them even survive, to various degrees, the pasteurization process (14, 36). However, one remarkable feature is the relative ease with which phages from these three species can propagate and reach high titers compared to the other lesser-known lactococcal phages. Undoubtedly, this basic fitness parameter explains their widespread distribution and recovery. Other lactococcal phage species are isolated more often from raw milk than from failed fermentations. These phages may not propagate efficiently in a factory environment, which is characterized by higher temperatures, rapid growth of would-be host strains, and mechanical constraints. For some of these species, it has also been difficult to obtain high titers in laboratory conditions, and plaques are usually very small. For example, lactococcal phages of the *Podoviridae* family (P034 and KSY1) have a long latent period that may explain their low titers (27, 50). Moreover, we observed large numbers of capsids without tails during our electron microscopic observations, indicating that phages with longer tails (949, P087, and 1706) were easily damaged (data not shown). Thus, like other viral communities, rare phages most probably serve as a "bank" and can become abundant in response to very specific environmental conditions (8). Then again, phages Q54 and 1358 are not isolated frequently but were easily amplified by using standard protocols. They may represent emerging groups. It will be interesting to analyze these phages in greater detail to gain a better understanding of their propagation and genetic makeup.

In this study, we used electron microscopic observations, Southern hybridization studies, and comparative sequence analyses (when complete genomes were available) to classify lactococcal phages into 10 groups. Phage classification has always been a subject of controversy (20, 31, 44, 48). For example, it has been suggested that P335 be split into three separate groups, namely, r1t, Sfi11, and Sfi21 (16, 46). This proposal was based on comparative genomics examining a structural gene module (capsid or tail) and on the study of phages active against *Streptococcus thermophilus* strains. Considering that P335-like phages are prone to genetic recombination through homologous recombination (6, 41) and that comparative complete genome analyses have illustrated the polythetic nature of this species, it may be premature to separate them into different groups. Moreover, despite the fact that *L. lactis* and *S. thermophilus* are used in dairy fermentation processes, they are very different bacterial species. *S. thermophilus* appears to be a species that has only recently emerged that has a clonal structure (21) and is sensitive to only one known polythetic species of temperate/lytic phages (10). In contrast, *L. lactis* strains are diverse, and as shown in this work,

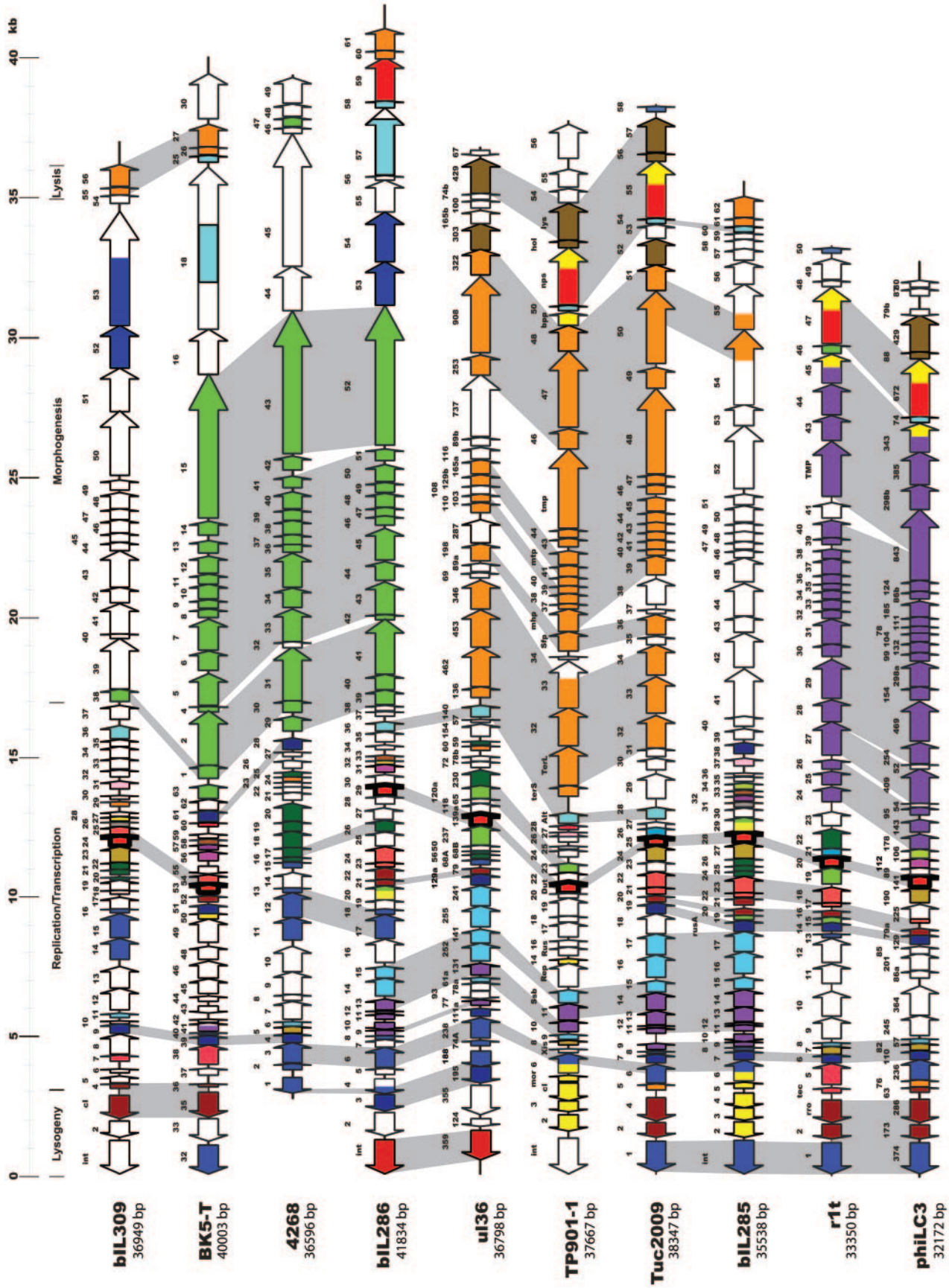


FIG. 3. Alignment of the genetic maps of 10 completely sequenced P335-like phages (updated from reference 30). Deduced proteins sharing >60% amino acid identity are represented using the same colors and linked with gray shading when possible. Open reading frames with unique sequences are displayed in white. The dUTPase genes are identified by thick lines.

they are sensitive to several genetically unrelated groups of phages. It thus seems a risky exercise to attempt to classify lactococcal phages by singling out one method as a universal taxonomic scheme (44, 54).

The revised classification of lactococcal phages and the improved multiplex PCR test presented in this work provide the basic knowledge and methodology needed to rapidly identify phages responsible for fermentation breakdowns and to follow the evolution of subdominant phages. This report also underscores the need to study the viruses in a given ecological niche as a whole by correlating phenotypic properties with genomic comparisons. Up to now, the only lactococcal phages characterized have been members of the three predominant groups. The characterization of rare phages will likely provide additional information on their biodiversity.

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